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The molecular basis for mefloquine resistance in the human malaria parasite, Plasmodium falciparum was investigated using recombinant DNA techniques. The parasites were found to be capable of manifesting extremely high levels of resistance to the drug in vitro. Resistant lines were found to be rapidly growing, impervious to various treatments, and significantly stimulated to grow by low levels of mefloquine. At the molecular levels, it was found that the genes for tubulin, a calmodulin-like protein, and a P-glycoprotein-like protein were all amplified in a quantitative relationship to the degree of resistance. Clones for each of these genes were isolated and characterized. Attempts were made to induce the amplification of tubulin genes in a sensitive parasite to see whether this event would confer resistance. High levels of vinblastine resistance were achieved but without evidence of gene amplification Hence, the exact relationship between the amplification event and drug resistance remains unresolved. A hypothetical relationship was developed to explain the amplification of these genes together possibly leading to the resistance trait.

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FOREWORD

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Stephen C. Merritt 90-03-22

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Stephen C. Merritt 90-03-22

Introduction

The studies conducted here were designed to identify the genetic events accompanying the emergence of the resistance trait in vitro and to define the nature of mefloquine resistance at the molecular level in parasites in which the trait has been induced and in human isolates demonstrating natural mefloquine resistance.

It is difficult to exaggerate the impact that the human malarias continue to have on the hundreds of millions of people living in endemic areas and the even greater risk which infection poses to individuals who must enter these regions without even the partial immunity conferred by previous exposure. Despite concerted efforts at vector control, chemoprophylaxis, and rapid diagnosis and treatment of suspected cases, the infection remains a risk to the health and well-being of roughly half of the world's population (1).

Of the four principal species of malarial parasites infecting humans, Plasmodium falciparum presents the most serious threat. Because of its potential lethality, this disease must be treated when detected, but the emergence and spread of drug resistant strains have increasingly prevented the use of what once were the most effective and reliable antimalarials. In addition, regions with overtly drug resistant strains often overlap with areas in which the Anopheline vectors of malaria have developed resistance to the most commonly used insecticides(2). While this has been occuring, significant progress has been made toward the identification of relevant protective antigens and the ability to produce these antigens by genetic engineering for the potential development of effective immunoprophylaxis. Undoubtedly, this approach holds a great deal of promise for the future, but until such vaccines can be developed and the logistical problems of testing, production, and deployment have been met, the other alternative, the derivation of new antimalarial drugs, will continue to play an essential role in practical malaria control (3).

The most promising member of the new generation of antimalarials is the synthetic quinolinemethanol derivative, mefloquine (WR 142,490), prepared during the intensive drug development program of the U.S. Army Medical Research and Development Command. This blood schizontocide, a structural analogue of quinine, is effective against many strains of Plasmodium falciparum resistant to other drugs and shows very low toxicity in humans (4). Mefloquine is also the only drug currently available for the treatment and prophylaxis of multidrug-resistant malaria and has recently received FDA approval for use in the U.S.

In nature, it is clear from previous studies that resistant parasites can exist in a clinical isolate in the absence of previous drug pressure and that these parasites can be selected by drug pressure to become the predominant population.

As with several other antimalarials, however, we know very little about the mode of action of mefloquine, less about the kinetics of the induction of resistance in vitro and in vivo, and virtually nothing about the molecular mechanisms which form the

basis of the resistance trait.

It is obvious that the induction of a resistant clone from a clonally-derived sensitive population requires a fundamental genetic difference between the two cell types. Conceivably, this difference could give rise to a transitory altered cell state or a relatively permanent change conferring resistance. In either case, however, changes in genetic expression or the genome itself leading to altered expression must be invoked to explain the difference. Even events such as post-translational modifications and peptide half-life differences must ultimately have a genetic basis.

The studies reported here were predicated on a simple hypothesis: the production of stable, heritable, mefloquine resistance in clones derived from a previously sensitive clone involves fundamental genetic changes which lead to altered gene expression conferring the resistance trait. These genetic changes would not have to lead to a continual expression of the resistance trait, but at the very least, they would have to provide significant changes in the parasites' ability to respond to drug pressure. The corollary of this hypothesis is that altered gene expression should be reflected in quantitative or qualitative changes in the products of the genes involved. Accordingly, these changes should be detectable as differences between sensitive and resistant cells at the molecular level providing that the means of detection is sufficiently sensitive. The detection of such differences should be possible by means of the specificity and sensitivity inherent in recombinant DNA techniques. The identification of gene products which differ will provide specific probes for the gene or genes which provide the mechanistic basis for resistance.

METHODOLOGY:

In the beginning of our analysis of W2 and W2MEF at the molecular level, we generated a genomic library for each clone using the bacteriophage vector Lambda gt11 (5). Our initial characterization of falciparum DNA with restriction endonuclease digestion indicated that conventional strategies for genomic cloning would likely not be adequate for our purposes. Specifically, the high A/T content of the DNA (approximately 81%) makes the use of enzymes often used for the fragmentation of genomic DNA for cloning less suitable. Accordingly, we used the enzyme Dra I which recognizes the restriction site, TTTAAA, and generates a blunt-ended fragment upon cutting. Although this enzyme recognizes a hexamer site, with the Plasmodium DNA, it cuts frequently (approximately every 220 nucleotides) and thus, acts like the 4-base restriction enzymes commonly used to fragment genomic DNA. The DNA from each clone was cut over varying time points with varying amounts of enzyme and the different fractions were pooled and subjected to size fractionation by stringent isopropanol precipitation and chromatography on Sephacryl S-1000. Fragments ranging between 2000 bp and 7000 bp were collected and methylated using Eco RI methylase. Synthetic Eco RI linkers were ligated to these fragments followed by Eco RI digestion and ligation into gt11.

The recombinant phage DNA was packaged into viable phages in vitro and the resulting libraries of recombinants were amplified on E. coli Y1090(r-) bacteria. Approximately 160,000 recombinants were present in the unamplified packaging mixtures. If we assume that the estimates of the plasmodial genome size are correct (approximately 2 X 10^7 base pairs) a random gene library consisting of 15,000 recombinant clones carrying 6 kb inserts should contain all clonable sequences with a probability of 99%. Our libraries contain ten times this number of clones and should, therefore, be representative of the genomes of the respective parasite clones. Although our choice of gt11 as a vector limits the size of the possible recombinant DNA insert (approximately 7.2 kb maximum), screening the larger number of clones did not represent a problem. In preliminary screening, 15,000 clones could be screened on a single 9 cm plate. For analysis of the genetic context of clones of interest we also generated librairies from both the sensitive and resistant parasite clones containing longer stretches of contiguous sequences in the phage vector EMBL 4 which accepts inserts up to 22 kb.

We also compared W2 and W2-MEF at the DNA level by direct restriction endonuclease digestion and agarose gel electrophoresis. No obvious differences were found between the two, but they were markedly different from other clones examined. This finding did not rule out the possibility of gene amplification being involved in the development of resistance, but it did indicate that if amplification occurs concommitantly with resistance it does not involve the generation of more than about 50 copies of the amplified sequence unlike the case in other drug resistant protozoa where amplified sequences can be directly visualized on gels (7).

We then did some preliminary screening of the libraries with radiolabeled genomic DNA (for repetitive sequences) and cDNA (for gene-specific sequences). Both libraries have comparable numbers of cDNA positive clones which have been collected into sub-libraries, but the W2MEF library appears to have roughly 10 times the number of repetitive sequence-specific clones when screened with W2MEF DNA. We know that some of these sequences differ between the two clones but we have not had time to do an in depth analysis of each of the roughly 200 clones isolated in this way. Instead, we concentrated on the few recombinant clones which we could demonstrate distinct quantitative differences in the resistant and sensitive parasites which appeared to correlate with the degree of resistance found in each (vide infra).

Subsequently,we also reapplied drug pressure to the resistant parasites in vitro to attempt to increase the level of resistance. This was done for two reasons. First, we wanted to begin to examine the limits of resistance that can be expressed to this drug. Secondly, we wanted to maximize our chances of detecting changes associated with resistance by increasing the overall resistance level. We were able to demonstrate that the parasite can adapt to as much as 2.4 ug/ml whereas the normal IC-50 for the sensitive parasite is about 5 ng/ml. We have kept the parasites under this level of continuous drug pressure for as long as 16 days with continued schizogony. We then began to

grow the parasites in quantities sufficient to allow the isolation of mRNA for cDNA cloning. We prepared the mRNA by oligo-d(T)-cellulose chromatography. This then was used for the preparation of two sets of cDNA libraries for cross-screening between the mefloquine sensitive clone W2 and the mefloquine resistant clone W2-MEF. One set was constructed using the bacteriophage expression vector gt11 while the other libraries were made using the vector gt10 (5). These two separate constructions were made to allow us to screen the libraries with either DNA probes or antibody to parasite proteins (gt11) or to ensure positive selection for recombinants (gt10). Both sets of libraries had active phage titers of greater than 1 million plaque-forming units per milliliter. This is at least a hundred times more than would be needed to represent even the least frequently expressed mRNA and hence all the libraries appear to be useful for our purposes. We have isolated DNA clones from the libraries representing alpha and beta tubulin and a calmodulin-like sequence.. These were isolated from the library using heterologous probes for the genes prepared from Trypanosoma gambiense from other studies in my laboratory.

These clones are of particular interest because we are beginning to suspect that both microtubules and calmodulin might be involved in the mechanisms behind at least certain aspects of drug resistance. During the Southern blotting experiments we determined that there are quantitative and qualitative changes in both the tubulin gene and the calmodulin gene dosage that appeared to correlate with the degree of resistance. In particular, we have compared three separate clones: D6 (naturally resistant to mefloquine but senstive to other antimalarials), W2 (sensitive to mefloquine but resistant to many other antimalarials), and MEF-2.4 (highly resistant to mefloquine and to most antimalarial drugs) . The blotting experiments indicated that as the degree of multidrug resistance increased the number of tubulin and calmodulin genes increased coordinately (Figure 2 and 3). Despite the use of heterologous probes, the signal strength of the clones isolated was very good even under stringent conditions (Figures 4 and 5). In addition, we have found that both tubulin genes and calmodulin are linked in DNA isolated from the parasite. We determined this by Southern blotting of genomic DNA as well as isolation of genomic recombinant clones using tubulin or calmodulin probes (Figures 6 and 7) In each case, clones positive for the tubulin probe were also recognized by the calmodulin probes under high stringency (Figure 8).

We have also done studies looking at the reversal of in vitro resistance with antimicrotubule agents (vinblastine and vincristine - ref. 9), calcium channel blockers (verapamil - ref 8), and calmodulin antagonists (calmodazolium and chlorpromazine ref. 9). Each of these agents was able to restore the mefloquine sensitivity to the resistant clone MEF-2.4 but less so to the more sensitive clone D6 or W2 (Figures 9, 10, and 11). We have found that this this clone grows faster than all of the other clones and strains which we maintain in the laboratory and that it is able to survive various treatments which are deleterious to other clones (heat shock, endotoxin, microtubule inhibitors, and calmodulin antagonists). Interestingly, although

highly resistant to quinine and moderately resistant to halofantrine, this line shows enhanced sensitivity to chloroquine (IC-50 of 26 ng/ml). Although this is still a measure of resistance, it is well below the level manifested by the parental line W2-MEF.

We have also found that calcium channel blockers, calmodulin antagonists, and microtubule inhibitors all reverse the resistance to mefloquine in this line in a manner similar to that reported by the WRAIR group for chloroquine. Since this pattern of reversal is very reminiscent of multi-drug resistance in other eukaryotic cells types, we began to examine the parasites for the presence of a 'P-glycoprotein-like' entity which might mediate the efflux response. Initial screening using a consensus sequence oligonucleotide indicated that there were sequences which bore structural similarity to the P-glycoprotein (9) and we isolated representative clones for further analysis. We wanted to use a molecular probe homologous to the P-glycoprotein to ask whether or not this gene (if present) is amplified coordinately with the manifestation of resistance. As we continued our work, it became clear that they were as were genes for the tubulins, alpha and beta isotypes, and a calmodulin-like sequence (Figure 12).

We have also placed the non-multidrug resistant clone D6 under vinblastine pressure <u>in vitro</u> and have obtained parasites which can survive high levels of this microtubule inhibitor. As we isolate lines which manifest increasing levels of resistance to this compound, we want to ask whether or not the lines will also be more resistant to known antimalarials in a manner similar to the multidrug resistant clones which have amplified the tubulin genes. This would help to confirm the participation of these genes in the development of resistance as well as the relative coordinate degree of amplification.

We have also continued the pressure on MEF-2.4 such that it can now survive at least 6 days of continuous pressure with 2.4 ug/ml. This is 480 times the IC-50 for the sensitive clone W2.

We then continued our study of mefloquine resistance in <u>Plasmodium falciparum</u> in addition to our examination of the multidrug resistance phenomenon. As noted above, we have detected an increase in the copy number for the alpha and beta tubulin genes and a calmodulin-like sequence. Previously, we have been referring to this latter gene as calmodulin since it shows strong hybridization characteristics with a trypanosome calmodulin-specific cDNA probe. We have now sequence about 500 bases of this gene and have yet to detect any sequence similarity with calmodulin. This is predictable however if, as in other systems, this cDNA clone with which we are working is flanked on both ends by long 5'- and 3'-non-coding regions (10). Since the probe recognizes only the coding domain of calmodulin, this would mean the probe is binding internally to the regions sequence which have been at both ends of the clone. Alternatively, this gene might not be calmodulin at all. It is interesting to note in this regard, that a sequence which is found amplified in human drug resistance cell lines, sorcin, is apparently a calcium-binding protease with substantial homology to calmodulin (11).

Regardless of the identity of this sequence, it is still clear that it is amplified in the more resistant malaria clones and that, as we reported earlier, it is truly contiguous with the two tubulin isotype genes. Thus, it remains of great interest even though we cannot yet identify it.

In an effort to facilitate this, we have sub-cloned unique fragments of genomic clones that are specific for alpha tubulin and beta tubulin, respectively. This should allow more rapid characterization of each gene's context in the genomic DNA.

The evidence that all three genes were contiguous and coordinately amplified suggested to us that one might be able to derive a multidrug resistant clone from a previously sensitive clone by selectively pressuring the parasites with agents which act on either microtubules or calmodulin as opposed to standard antimalarials. If the pressured gene were amplified in response to the presence of the agent, then the entire locus of genes should be co-amplified in a sub-set of the clones. If this confers multidrug resistance to a previously sensitive cell population, one can inferentially implicate these genes in the manifestation of resistance.

Accordingly, we maintained clone D6 under increasing vinblastine pressure, since this agent is highly toxic to the parasites and since it binds preferentially to the microtubules causing their disassembly. We now have a cell line which can withstand 5X the normal IC-50 for this compound. Much like our observations of the highly mefloquine resistant clone, this line grows better in vitro, tends toward large vacuole formation, and appears to have lost or suppressed the capacity for gametocyte production.

Our vinblastine resistant clone has been examined in more detail and we have characterized the nature of the resistance. Rather than simply surviving the pressure of the drug in short bursts with long recovery times, we now have a population which recovers in about a week from levels of this agent that should kill the entire population. The parasites appear to be very healthy and they grow remarkably well compared to the progenitor clone D6 which tends to be somewhat fastidious. This is important in the context of resistance to current or future antimalarials even though this compound is not used clinically for the infection. This agent usually acts on Plasmodium in a very toxic and irreversible manner. With sensitive cells, once you have applied the drug in vitro, you cannot remove the toxicity by washing or pulsing with vinblastine analogues. Thus, it is very clear that the parasites have the genetic and biochemical means to overcome the metabolic lesions induced by even highly toxic compounds.

These studies have been encouraging in terms of deriving a resistant line, but the results of our drug testing in vitro have all but disproved our original hypothesis behind the derivation of this vinblastine resistant population. After testing the resistant parasites in the standard radioactive hypoxanthine uptake assay, we have determined that the cells remain sensitive to all the antimalarials tested. In addition, the measured IC-50's are within the range expected for the parental clone D6. This could be explained in a couple of ways although we do not

know what the reason is. First, the resistant parasites could form a minority subset of the entire population and in the absence of pressure, their numbers may diminish substantially. This would reduce the population IC-50 even though resistant parasites are present. It is also possible that the resistant in this new line is not based on tubulin gene amplification. All it would take would be one or a series of point mutations which substantially reduced the binding of the drug to the microtubules. This has been reported in yeast beta tubulin sequences. It is also possible that there are two pumps for the different drugs or two binding sites on a P-glycoprotein-like molecule. Changes leading to accelerated efflux of vinblastine may not affect the way in which the cell handles other agents.

We have also analyzed the amplified genes in the multidrug resistant parasites comparatively with the sensitve clones. It is evident that one or more genes with sequence homology to the 3'-most end of the P-glycoproteins of mouse, human, and hamster are amplified in these resistant clones. The degree of amplification parallels that seen with the other genes which are amplified and the quantitative pattern is the same. We believe, but have not proven, that at least one of these P-glycoprotein-like sequences is contiguous with the tubulins and calmodulin-like sequence. If this is correct, we have probably detected an 'amplicon' which is selectively increased in copy number in response to drug pressure. This makes the idea of intracellular cooperation of these elements in the manifestation of resistance even more inviting. It might also prove to be an appropriate region for which to probe by the polymerase chain reaction or conventional hybridization for a quantitative estimate of drug resistance.

Summary and Conclusions

The problem of drug-resistant malaria is an acute one from the military perspective. The absence of effective prophylaxis and treatment represents a major consideration affecting decisions about troop and personnel deployment into endemic areas even for short periods of time. Most of the potentially volatile areas of the world where the necessity for a U.S. military presence might arise are malarious zones as are many other regions where advisory and support personnel are presently situated or may be stationed in the future. The spreading prevalence of chloroquine resistance concommitantly with resistance to other antimalarials may necessitate the increased clinical use of mefloquine in these situations, but the parasites we will use in these studies illustrate something of the potential for widespread resistance to this drug as well. Certainly, information about the mechanisms, potential, and extent of resistance would be significant for the future use of this drug and as fundamental information about the molecular strategies employed by the parasites to circumvent the toxic actions of antimalarials in general.

Our results strongly indicate that the parasites have the capacity to develop very high levels of resistance to various agents and that multidrug resistance as a phenomenon similar to that seen with mammalian cells appears to be present in some

parasite lines. As noted in previous reports on this work, the resistant parasite lines which we have derived are exceedingly hardy. They will outgrow any other strain or line which we have in culture in our laboratory and they will withstand environmental assaults (accidental heat shock, endotoxin treatment, etc) more readily than other parasite lines. In addition, these lines appear to be stimulated by low levels of mefloquine. This stimulation results in an overall increase in the number of parasites but we do not know whether this is a change in division rate, a shortened cell cycle, enhanced efficiency of red cell penetration, or the like. What is clear however, if one can extrapolate from these findings to what might occur in the field setting, parasite strains might arise which are resistant to prophylactic and therapeutic levels of this drug, which may be harder to treat once established because of their overall hardiness, and which may be stimulated to faster development by low levels of the drug as might be achieved by chemoprophylactic use. This is especially likely given that naturally resistant strains have been detected in various parts of the world. One potentially positive aspect of this work is the finding that the parasite appear in some cases to develop collateral sensitivity to certain agents upon the development of high levels of mefloquine resistance (particularly, chloroquine and gentamycin).

The discovery of gene amplifications which appear to correlate with the degree of resistance suggests that some degree of coordinate activity among the various genes products may lead to the manifestation of the resistance trait. One could envision, for example, the interplay between vesicular transport within the cell along microtubule-defined paths, calcium-mediated enzyme activities, and a p-glycoprotein-like activity leading to compartmentalization of the drugs within the cell limiting their toxicity and ultimately leading to the enhanced efflux of drugs from the cell surface.

In addition, although the specific application to malaria remains speculative, from these studies it may be possible to develop either DNA probes or antibodies specific for the resistance trait allowing for the rapid identification of the drug-resistance trait in cells without the necessity for culture. If any or all of these genes turns out to be significantly and consistently amplified in resistant cells, it may be possible to use the gene we have identified to develop a rapid diagnostic procedure for the presence of the resistance trait. If the components of the mult-drug resistance network are expressed on the surface of infected cells as they appear to be in tumor cells, it may also be possible to develop humoral or cellular immunity to these components by vaccination. Thus those parasites not responding to chemotherapy my be cleared more efficently by immune mechanisms than would normally be the case. This might provide the 'edge' required by the host to curtail the development of the infection and in so doing, modify its lethality if not eliminating the parasites altogether.

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FIGURE 1

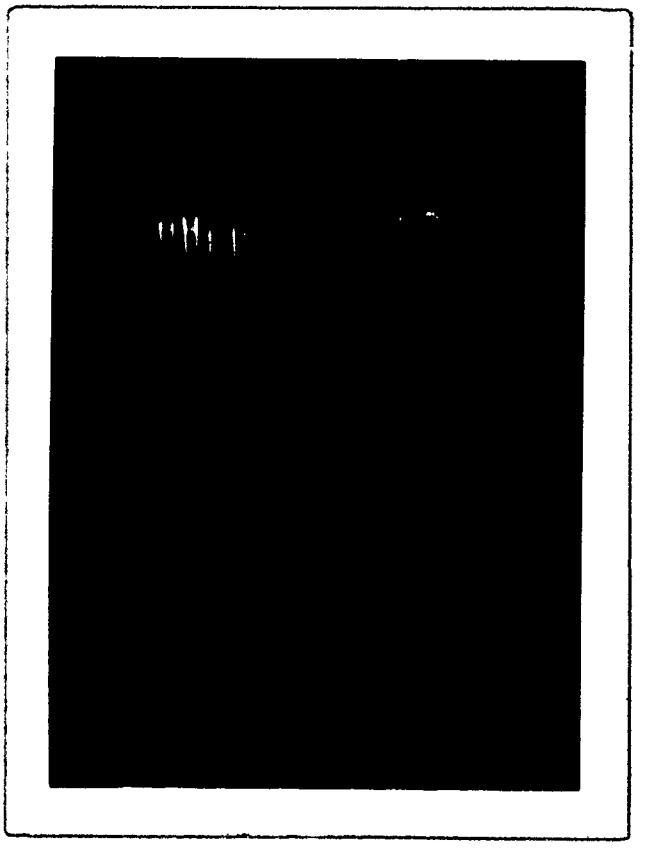
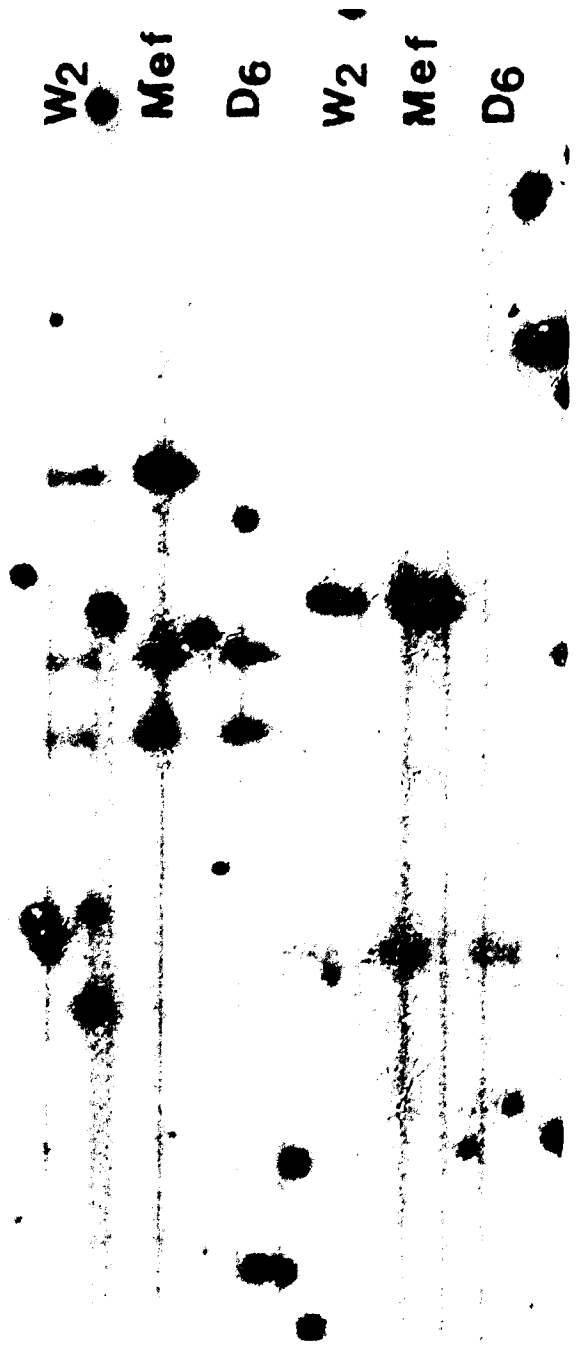


Figure 1. Ethidium bromide stained gel comparing W2 and W2-MEF.
Lane 1 and Lane 14 - MW marker at 1 kilobase intervals
Lane 2 and Lane 3 - W2 and W2-MEF respectively cut with Alu I
Lane 4 and Lane 5 - W2 and W2-MEF respectively cut with Bam HI
Lane 6 and Lane 7 - W2 and W2-MEF respectively cut with Dra I
Lane 8 and Lane 9 - W2 and W2-MEF respectively cut with Eco RI
Lane 10 and Lane 11 - W2 and W2-MEF respectively cut with Hind III
Lane 12 and Lane 13 - W2 and W2-MEF respectively cut with Pst I

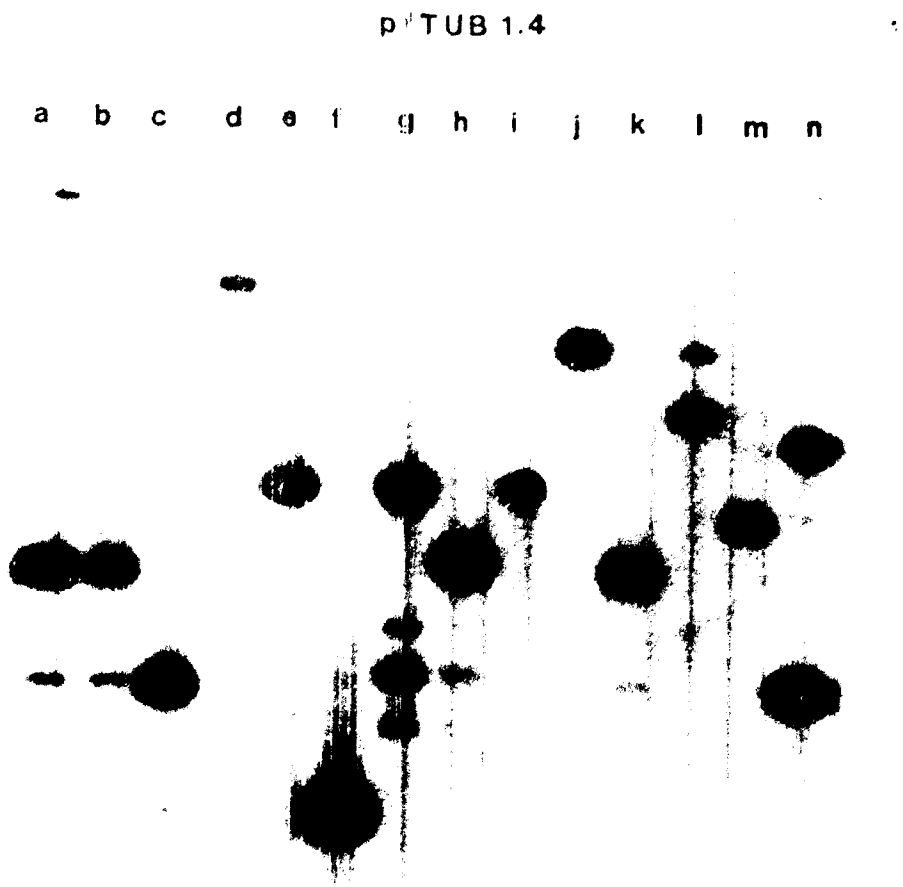
FIGURE 2

Southern blot of falciparum genomic DNA of parasite clone W2, W2-Mef, and D6. Blot was probed with α-tubulin-specific 1140 bp Kpn-Eco RI fragment of trypanosome tubulin repeat. Lanes 1, 2, and 3 were digested with Dra I while Lanes 4, 5, and 6 were cut with Mbo I.

FIGURE 3

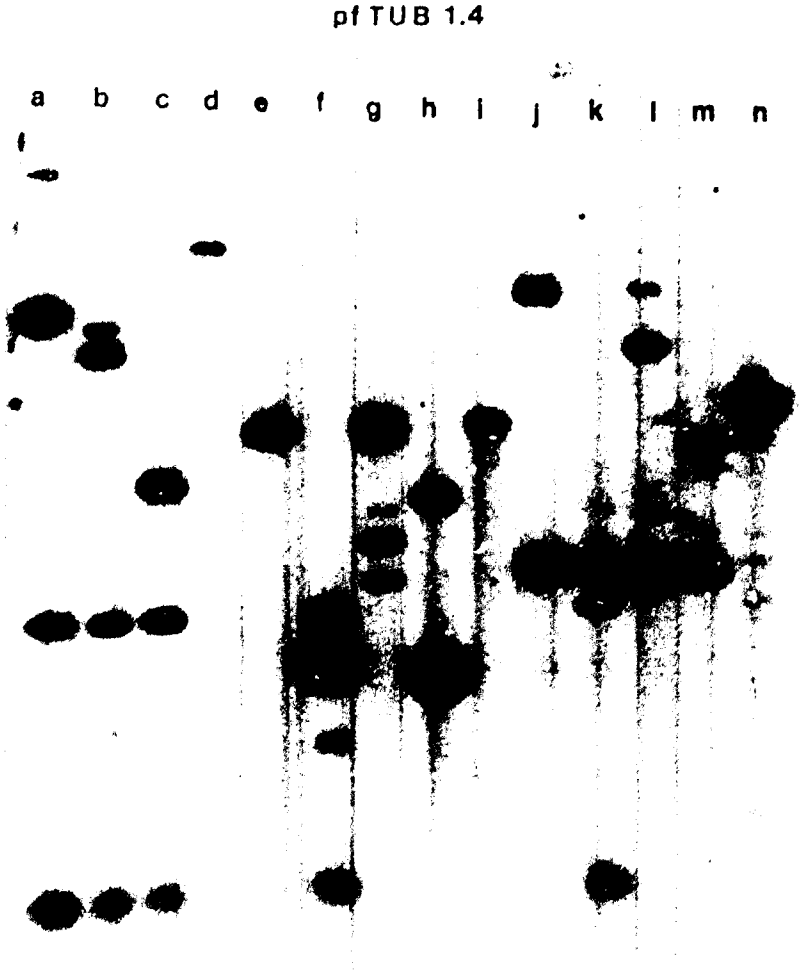


Southern blot of falciparum genomic DNA of parasite clones W2. W2-Mef. and D6. Blot was probed with α-tubulin-specific 970 bp trypanosome subclone. Lane 1. 2 and 3 were Dra I digests while Lanes 4. 5. and 6 were cut with Eco RI and Ssp I.



Southern of recombinant phage pfTUB 1.4 probed with nick-translated Kpn I/Eco RI fragment of pTB81 specific for a-tubulin. The restriction digestions are as follows:

Lane a.	Bgl II	Lane h.	Eco RI/Hind III
Lane b.	Bgl II/Dra I	Lane i.	Eco RI/Ssp I
Lane c.	Bgl II/Ssp I	Lane j.	Hind III
Lane d.	Dra I	Lane k.	Hind III/Bgl II
Lane e.	Eco RI	Lane l.	Hind III/Dra I
Lane f.	Eco RI/Bgl II	Lane m.	Hind II/Ssp I
Lane g.	Eco RI/Dra I	Lane n.	Sin I



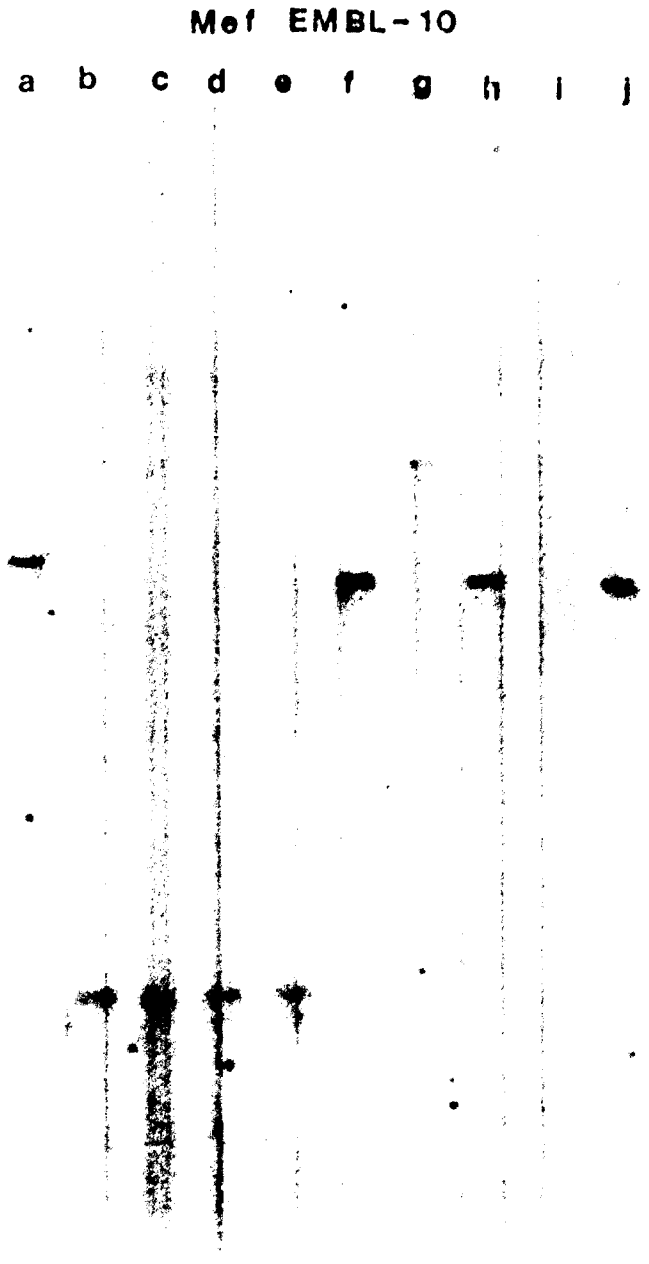
Southern of recombinant phage pfTUB 1.4 probed with nick-translated pβ2, a subclone containing the 970 bp Bgl II fragment of pTB81. The restriction digestions are as follows:

Lane a.	Bgl II	Lane h.	Eco RI/Hind III
Lane b.	Bgl II/Dra I	Lane i.	Eco RI/Ssp I
Lane c.	Bgl II/Ssp I	Lane j.	Hind III
Lane d.	Dra I	Lane k.	Hind III/Bgl II
Lane e.	Eco RI	Lane l.	Hind III/Dra I
Lane f.	Eco RI/Bgl II	Lane m.	Hind II/Ssp I
Lane g.	Eco RI/Dra I	Lane n.	Sin I

FIGURE 5

Recombinant genomic clone Mef EMBL-10 blotted to nitrocellulose and probed with nick-translated pTB8i containing trypanosome β- and a-tubulin genes.

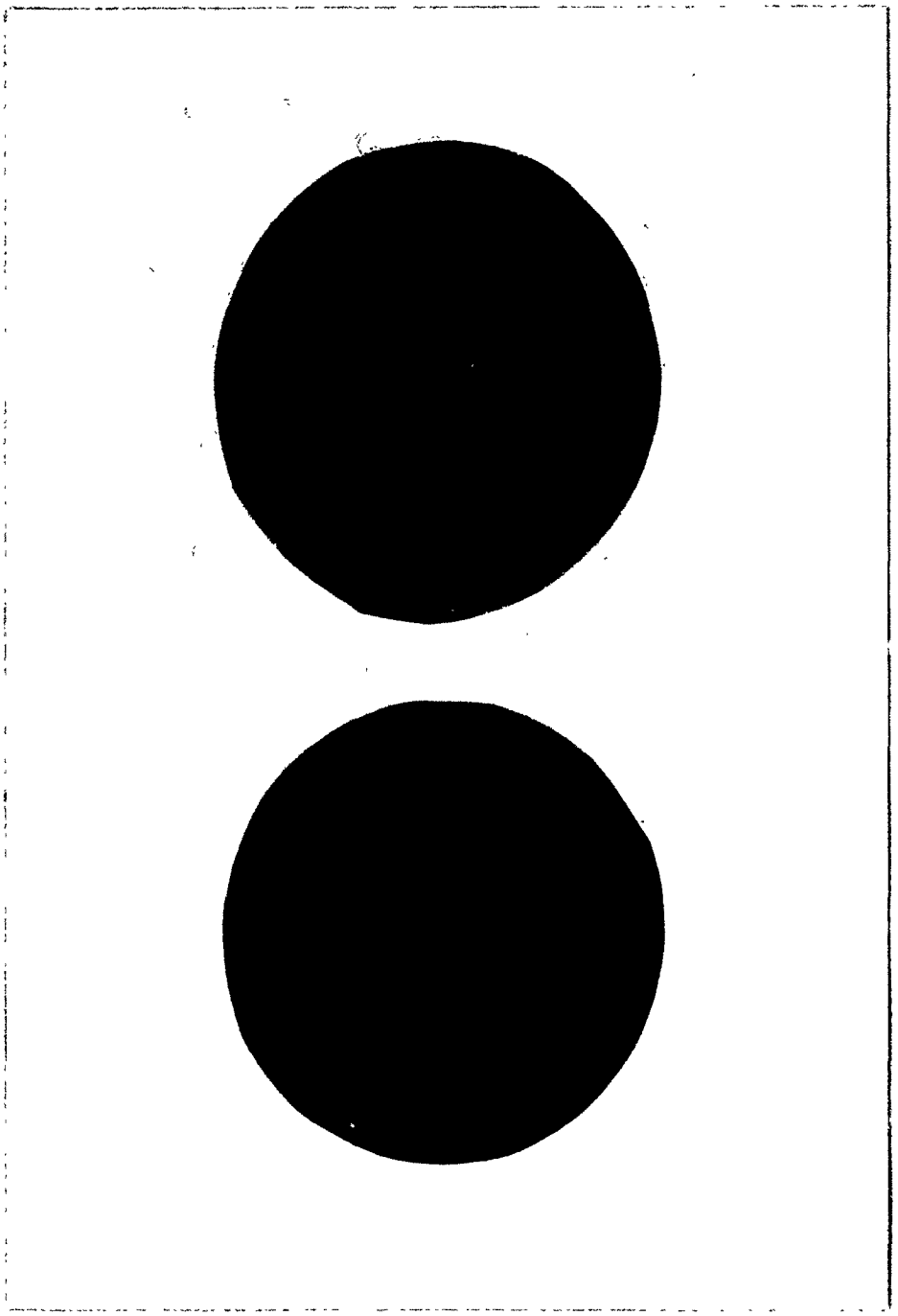
Lane a. Bgl II
Lane b. Eco RI
Lane c. Hind II
Lane d. Kpn I
Lane e. Sma I



Southern blot analysis of clone Mef EMBL-10 with 900 bp *P. falciparum* cDNA clone selected with trypanosome calmodulin probe. Restriction digestions were as follows:

Lane a.	Bam HI	Lane g.	Hind III
Lane b.	Bgl II	Lane h.	Kpn I
Lane c.	Eco RI	Lane i.	Kpn I/Hind III
Lane d.	Eco RI/Bam HI	Lane j,	Sac II
Lane e.	Eco RI/Hind III	Lane k.	Sac II/Hind III
Lane f.	Eco RI/Ssp I		

FIGURE 8



Duplicate nitrocellulose filters of recombinant phage pfTUB 1.4. Replica filter pfTUB 1.4a was screened with nick-translated pTV23 specific for β- and α-tubulin and filter pfTUB 1.4b was screened with nick-translated pCAL1, a cDNA from P. falciparum with strong sequence similarity to calmodulin.

FIGURE 9

ACTIVITY OF MODULATION AGENTS ON RESPONSE OF CLONE D6 TO ANTIMALARIALS -
IN VITRO MICRODILUTION ASSAY ASSESSMENT OF REVERSAL

DRUG	ALONE	+ VERAPAMIL (1 uM)	+ CALMODIZOLIUM (0.5 uM)	+ VINBLASTINE (50
	(FIFTY PERCENT INHIBITORY CONCENTRATIONS BASED ON HYPOXANTHINE UPTAKE) NG/ML			
CHLOROQUINE	2.1	2.4	1.9	2.3
MEFLOQUINE	26.7	25.9	25.0	26.8
AMODIAQUINE	0.89	0.92	ND*	ND
QUININE	10.1	9.5	8.5	9.7
QUINACRINE	12.3	13.4	ND	ND

* NOT DETERMINED

FIGURE 10

ACTIVITY OF MODULATION AGENTS ON RESPONSE OF CLONE W2 TO ANTIMALARIALS -
IN VITRO MICRODILUTION ASSAY ASSESSMENT OF REVERSAL

DRUG	ALONE	+ VERAPAMIL (1 uM)	+ CALMODIZOLIUM (0.5 uM)	+ VINBLASTINE (50 nM)
	(FIFTY PERCENT INHIBITORY CONCENTRATIONS BASED ON HYPOXANTHINE UPTAKE) NG/ML			
CHLOROQUINE	90.3	16.8	28.4	25.3
MEFLOQUINE	4.5	5.1	4.3	4.1
AMODIAQUINE	2.8	1.1	ND*	ND
QUININE	63.5	17.4	21.6	44.2
QUINACRINE	107.6	27.9	ND	ND

* NOT DETERMINED

FIGURE 11

ACTIVITY OF MODULATION AGENTS ON RESPONSE OF CLONE W2MEF-2.4 TO ANTIMALARIALS - IN VITRO MICRODILUTION ASSAY ASSESSMENT OF REVERSAL

DRUG	ALONE	+ VERAPAMIL (1 uM)	+ CALMODIZOLIUM (0.5 uM)	+ VINBLASTINE
	(FIFTY PERCENT INHIBITORY CONCENTRATIONS BASED ON HYPOXANTHINE UPTAKE) NG/ML			
CHLOROQUINE	46.8	11.0	17.1	23.4
MEFLOQUINE	29.0	6.8	6.2	6.9
AMODIAQUINE	3.2	1.2	ND*	ND
QUININE	78.4	14.9	23.5	25.7
QUINACRINE	111.7	31.7	ND	ND

* NOT DETERMINED

FIGURE 12

DENSITOMETER SCANNING DETERMINATION OF ESTIMATED GENE COPY NUMBER FOR PLASMODIAL GENES IN DRUG SENSITIVE AND MULTIDRUG RESISTANT CLONES - INTERCLONAL VARIATION IN RELATIVE NUMBER

PARASITE CLONE	RELATIVE AUTORADIOGRAM BAND INTENSITIES* (SINGLE NYLON SOUTHERN) ALPHA TUBULIN	BETA TUBULIN	CALMODULIN	P-GLYCOPROTEIN
D6	1.05	1.15	1.1	1.0
W2	3.4	3.1	1.6	1.8
W2MEF-2.4	6.1	6.3	3.4	3.7

* SCANNING WAS CARRIED OUT ON AUTORADIOGRAMS USING AN E-C APPARATUS CORP. DENSITOMETER WITH INTEGRATOR. BECAUSE OF DIFFERENCES IN PROBE LENGTH AND SPECIFIC ACTIVITY, ONLY INTERCLONAL COMPARISONS FOR THE SAME GENE ARE VALID. THESE VALUES DO NOT REPRESENT THE ABSOLUTE COPY NUMBER PER GENOME EQUIVALENT ALTHOUGH THERE ARE PROBABLY 2 ALPHA AND TWO BETA TUBULIN GENES IN EACH HAPLOID NUCLEUS (UNPUBLISHED OBSERVATIONS).